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Figures and figure supplements

A network of heterochronic genes including *Imp1* regulates temporal changes in stem cell properties

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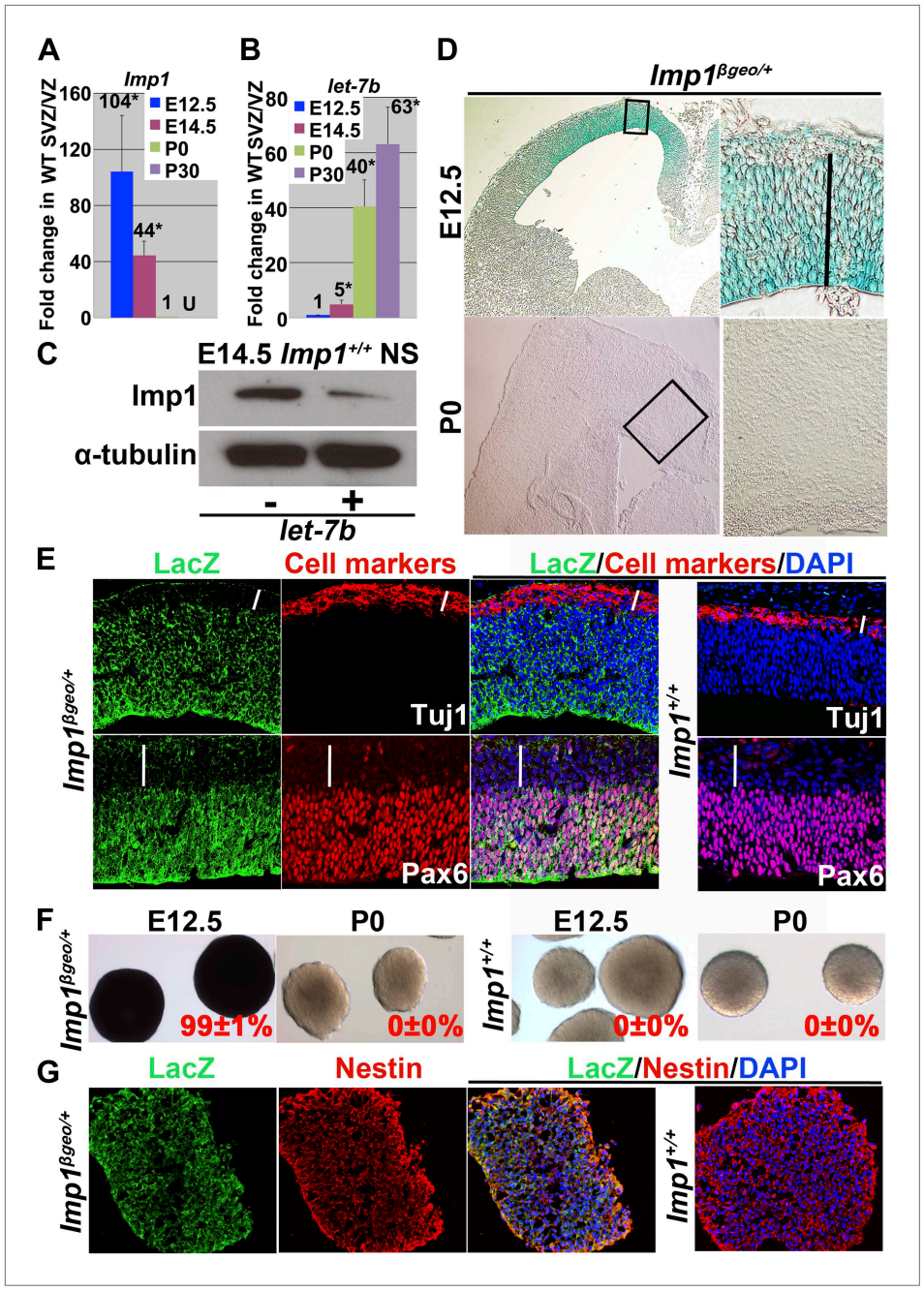

Figure 1. *Imp1* expression declines over time in neural stem/progenitor cells in the dorsal telencephalon and is extinguished postnatally. (**A** and **B**) qPCR for *Imp1* (**A**) and *let-7b* (**B**) in E12.5 dorsal telencephalon, E14.5 dorsal telencephalon, P0 lateral ventricle VZ/SVZ, and P30 lateral ventricle VZ/SVZ (fold change mean±SD for 3–4 mice/stage; U, not detectable above background; *p<0.01). (**C**) Western blot of E14.5 wild-type neurospheres infected with either *GFP-only* control lentivirus (−) or with *let-7b+GFP* lentivirus (+). *Let-7b* overexpression reduced IMP1 expression. (**D**) X-gal staining of sections from E12.5 and P0 *Imp1*$^{\beta\text{-geo/+}}$ forebrain. *Imp1* was expressed in a medial-high/lateral-low gradient in the E12.5 dorsal telencephalon and confined to undifferentiated cells in the VZ/SVZ (solid line). At P0, no X-gal staining was detectable. A high magnification image is shown for the boxed area on the low magnification image to the left. See also ***Figure 1—figure supplement 1A,B*** for X-gal staining in E10.5, E14.5, and E16.5 brains. (**E**) Immunostaining for LacZ in the dorsal telencephalon from E13.5 *Imp1*$^{\beta\text{-geo/+}}$ and *Imp1*$^{+/+}$ mice. *Imp1* was expressed by the Pax6+ neural progenitors in the VZ but not by the TuJ1+ neurons at the cortical plate (white bars). Nuclei were visualized using 4′6-diamino-2-phenylindole dihydrocloride (DAPI) staining. (**F**) Virtually all neurospheres cultured from E12.5 *Imp1*$^{\beta\text{-geo/+}}$ dorsal telencephalon, but not from P0 *Imp1*$^{\beta\text{-geo/+}}$ neocortical VZ,

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stained with X-gal (mean ± SD % X-gal+, three experiments). (**G**) LacZ and Nestin immunostaining overlapped in sections through neurospheres cultured from *Imp1*$^{\beta\text{-geo}/+}$ E12.5 dorsal telencephalon.

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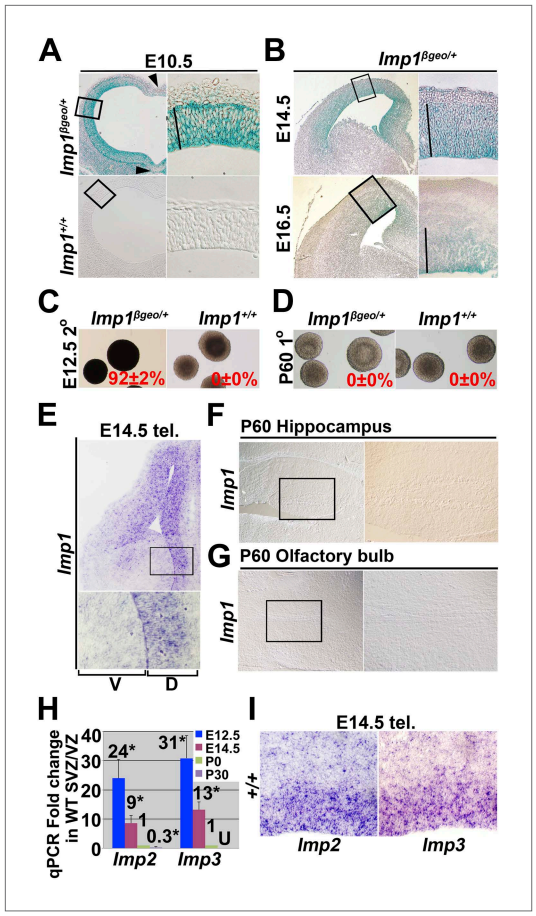

Figure 1—figure supplement 1. *Imp1* expression in the fetal brain is spatially restricted over time and extinguished in adult brain. (**A**–**B**) X-gal staining of sections from the E10.5 *Imp1*$^{\beta\text{-}geo/+}$ or wild-type telencephalon (**A**) and E14.5 or E16.5 *Imp1*$^{\beta\text{-}geo/+}$ forebrains (**B**). *Imp1* was expressed throughout the dorsal and ventral telencephalon at E10.5 except for the floor plate and roof plate (arrowheads). At E14.5 and E16.5, *Imp1* was expressed in a medial-high/lateral-low gradient in the dorsal telencephalon and confined to the undifferentiated cells in the VZ/SVZ (solid lines). Higher magnification images of the boxed areas are shown to the right of each low magnification image. (**C** and **D**) X-gal staining was maintained in most secondary neurospheres upon passaging of E12.5 *Imp1*$^{\beta\text{-}geo/+}$ dorsal telencephalon-derived primary neurospheres (**C**). No X-gal staining was detected in primary neurospheres cultured from P60 *Imp1*$^{\beta\text{-}geo/+}$ SVZ (**D**). (mean ± SD % X-gal+, three experiments). (**E**–**G**) In situ hybridization for *Imp1* on coronal sections of E14.5 telencephalon (**E**), P60 hippocampus (**F**), or sagittal sections of P60 olfactory bulb (**G**). *Imp1* transcripts were detected in the dorsal region of the telencephalon but not in neurogenic regions of the adult brain. Higher magnification images reflect the boxed regions in each low magnification image. (**H**) qPCR for *Imp2* and *Imp3* in E12.5 dorsal telencephalon, E14.5 dorsal

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telencephalon, P0 lateral ventricle VZ/SVZ, and P30 lateral ventricle VZ/SVZ (fold-change mean ± SD for 3–4 mice/stage; U, not detectable above background; $p<0.01$). (**I**) In situ hybridization for *Imp2* and *Imp3* in E13.5 dorsal telencephalon sections.

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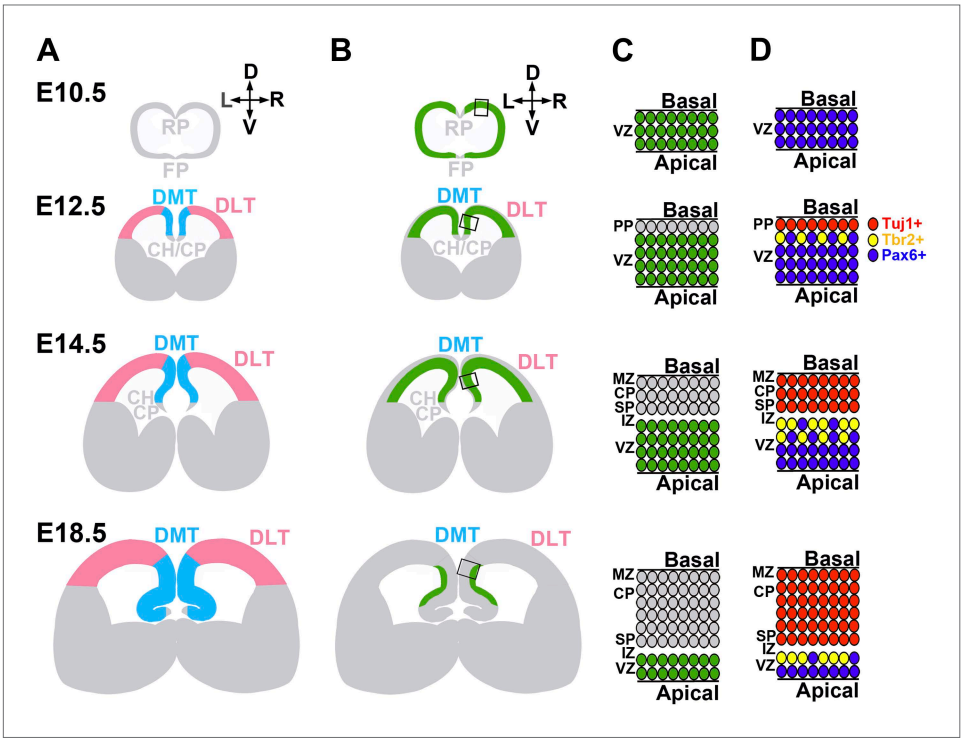

Figure 1—figure supplement 2. Schematic showing fetal telencephalon development and *Imp1* expression. (**A**) Anatomy during fetal telencephalon development (D: dorsal, V:ventral, L:left, and R:right). At E10.5, two signalling centers, floor plate (FP) and roof plate (RP) exist in the ventral and dorsal telencephalon. At E12.5 and later, dorsal telencephalon can be divided into dorsomedial telencephalon (DMT, light blue) and dorsolateral telencephalon (DLT, pink). Choloid plexus (CP) and cortical hem (CH) constitute the dorsal end of telencephalon. (**B**) Regions where *Imp1* was expressed are indicated in green. At E10.5, *Imp1* was expressed throughout the forebrain, except in the floor plate and roof plate. At E12.5, Imp1 was expressed typically in undifferentiated cells in the dorsal telencephalon but not in ventral telencephalon or in basal neurons. At E14.5 and later, *Imp1* expression was gradually confined to the apical region of the dorsomedial telencephalon. (**C** and **D**) Schematics show a close up of the boxed regions in panel **B** to illustrate the cellular layers during cortical development and the cells that expressed *Imp1* in those layers. At E10.5, undifferentiated Pax6+ neural stem cells (blue ovals in **D**) dominate all layers of the developing telencephalon and all cells express Imp1 (green in **C**). At E12.5, neural stem cells start to differentiate into Tbr2+ intermediate neural progenitors (yellow ovals in **D**) and Tuj1+ neurons (red ovals in **D**) that form the preplate (PP in panel **C**). At E14.5 and E18.5, newly formed neurons constitute the marginal zone (MZ), cortical plate (CP) and subplate (SP) on the basal side of the developing cortex. The intermediate zone (IZ) is a cell sparse region that lies between the SP and VZ. *Imp1* is expressed in undifferentiated cells at these stages (green ovals in **C**).

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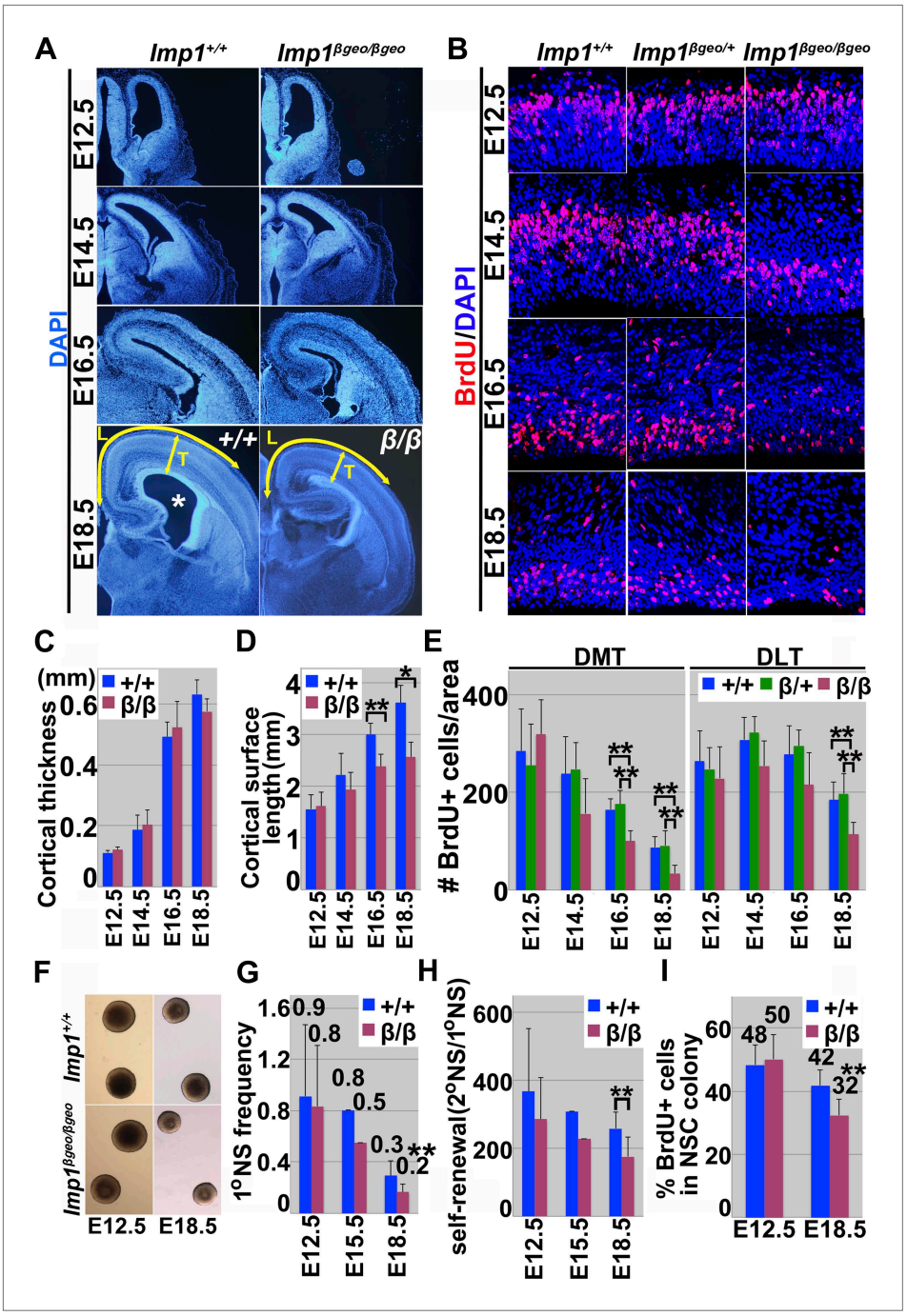

Figure 2. *Imp1* deficiency reduces brain size and pallial expansion due to reduced proliferation of fetal neural stem/progenitor cells. (**A**) Coronal sections of *Imp1*$^{+/+}$ and *Imp1*$^{β\text{-}geo/\ β\text{-}geo}$ telencephalons. The lateral ventricle is indicated with an asterisk in the *Imp1*$^{+/+}$ brain. Morphological abnormalities were visible in the *Imp1*$^{β\text{-}geo/β\text{-}geo}$ telencephalon as early as at E14.5. Pallial expansion was impaired and the lateral ventricle collapsed in the *Imp1*$^{β\text{-}geo/\ β\text{-}geo}$ brain at E18.5. The length (L) and thickness (T) of the pallial regions are indicated with yellow arrows. (**B**) Dorsomedial telencephalon (DMT) sections from E12.5, E14.5, E16.5 or E18.5 *Imp1*$^{+/+}$ *Imp1*$^{β\text{-}geo/+}$ or *Imp1*$^{β\text{-}geo/β\text{-}geo}$ embryos were stained with an antibody against BrdU. Reduction of cell proliferation was apparent in *Imp1*$^{β\text{-}geo/\ β\text{-}geo}$ telencephalon at E16.5 and E18.5. (**C**–**D**) The length (L) of the pial surface from the pallial/subpallial boundary to the retrosplenial cortex (L in panel **A**) was significantly shortened in the E16.5 and E18.5 *Imp1*$^{β\text{-}geo/β\text{-}geo}$ telencephalon (**p<0.05,*p<0.01; four brains/genotype), but cortical thickness (T) was not significantly affected. (**E**) BrdU immunostaining revealed a significant reduction in the frequency of proliferating cells in E16.5 and E18.5

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Imp1$^{\beta\text{-geo}/\beta\text{-geo}}$ telencephalon but not at E14.5 or E12.5 (**p<0.05; mean ± SD for 3–5 brains/genotype/stage and 6–8 sections/brain). The reduction was more prominent in DMT, where *Imp1* was more strongly expressed, relative to dorsolateral telencephalon (DLT). (**F**) Typical neurospheres after 8 days culture from E12.5 or E18.5 dorsal telencephalon cells dissociated from wild-type or *Imp1*$^{\beta\text{-geo}/\beta\text{-geo}}$ mice. (**G**–**H**) *Imp1* deficiency significantly reduced the percentage of cells that formed multilineage neurospheres and their self-renewal potential (the number of cells from individual primary neurospheres that formed multilineage secondary neurospheres upon subcloning), at E18.5 but not at E12.5 (**p<0.05; mean ± SD for 5–6 experiments/stage). We observed lower frequency and self-renewal potential of *Imp1* deficient multipotent neurospheres in two experiments performed at E15.5. (**I**) At E18.5, but not at E12.5, the percentage of cells within *Imp1*$^{\beta\text{-geo}/\beta\text{-geo}}$ multilineage colonies that incorporated a 20 min pulse of BrdU was significantly lower than in *Imp1*$^{+/+}$ colonies (three independent experiments/stage; **p<0.05).

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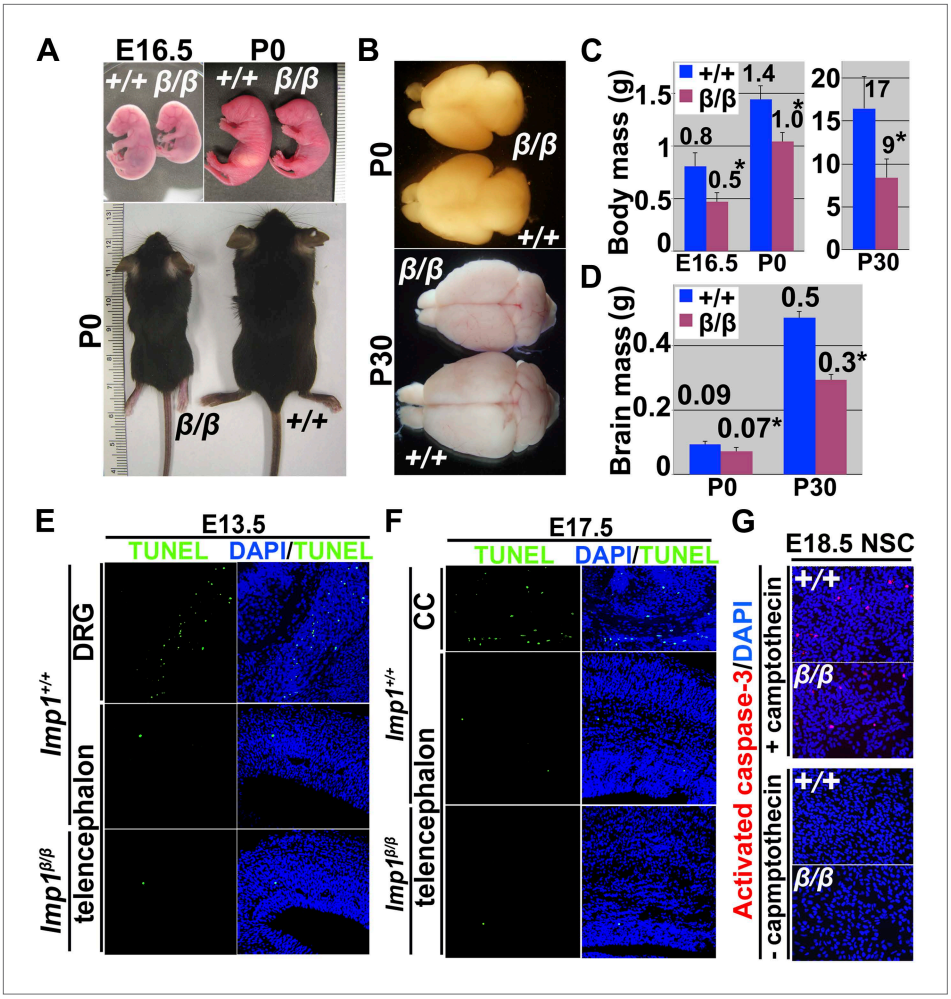

Figure 2—figure supplement 1. *Imp1*-deficient mice exhibit growth retardation and reduced brain mass but not increased cell death. (**A**) *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) mice were smaller than wild-type littermates (+/+) at E16.5, P0, and P30. (**B**) *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) brains were smaller than wild-type brains (+/+) at P0 and P30. (**C**) *Imp1*$^{β\text{-}geo/β\text{-}geo}$ mice had significantly reduced body mass relative to wild-type controls (8–14 embryos at E16.5, 7–12 mice at P0, and 6–10 mice at P30; *p<0.01; error bars represent SD). (**D**) *Imp1*$^{β\text{-}geo/β\text{-}geo}$ brains (β/β) were significantly smaller than wild-type controls (+/+) at P0 and at P30 (*p<0.01; 13–14 brains/genotype at P0, and 6–10 at P30). (**E**–**G**) To monitor apoptotic cell death, sections from the dorsal telencephalon of wild-type or *Imp1*$^{β\text{-}geo/β\text{-}geo}$ mice at E13.5 (**E**) or E17.5 (**F**) were subjected to TdT-mediated dUTP nick end labeling (TUNEL). Nuclei were visualized with DAPI staining. TUNEL positive cells (green) were rare in dorsal telencephalon irrespective of genotype, in contrast to E13.5 dorsal root ganglia (DRG) or E17.5 corpus callosum (CC; positive controls) where apoptotic cells were common. Activated caspase-3 positive dying cells were also rare within E18.5 neural stem cell colonies irrespective of genotype (lower two images) (**G**). Some cultures were treated with camptothecin to induce apoptosis (positive controls, upper two images).

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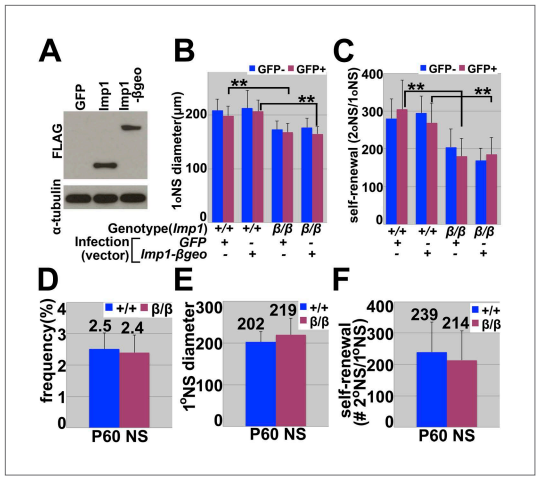

Figure 2—figure supplement 2. *Imp1* deficiency, but not *Imp1-βgeo* overexpression, reduced fetal (but not adult) neural stem cell self-renewal. (**A-C**) E18.5 wild-type (+/+) or *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) dorsal telencephalon cells were infected with *GFP-only* control retrovirus (*GFP*) or 3XFLAG-*Imp1-βgeo-GFP* retrovirus (*Imp1-βgeo*). *Imp1* deficiency, but not the over-expression of the 3XFLAG-βgeo fusion protein, significantly reduced neurosphere size (**B**) and self-renewal (**C**). (**p<0.05; mean ± SD for three experiments). Note that the *Imp1-βgeo* fusion construct did not contain the *Imp1* 3′ UTR that has the *let-7* binding sites and therefore would not be expected to be influenced by *let-7* expression. (**D**–**F**) *Imp1* deficiency did not affect the percentage of cells cultured from P60 SVZ that formed multilineage neurospheres (**D**), neurosphere size (**E**), or self-renewal potential (the number of cells from individual primary neurospheres that formed multilineage secondary neurospheres upon subcloning) (**F**; mean ± SD for three experiments).

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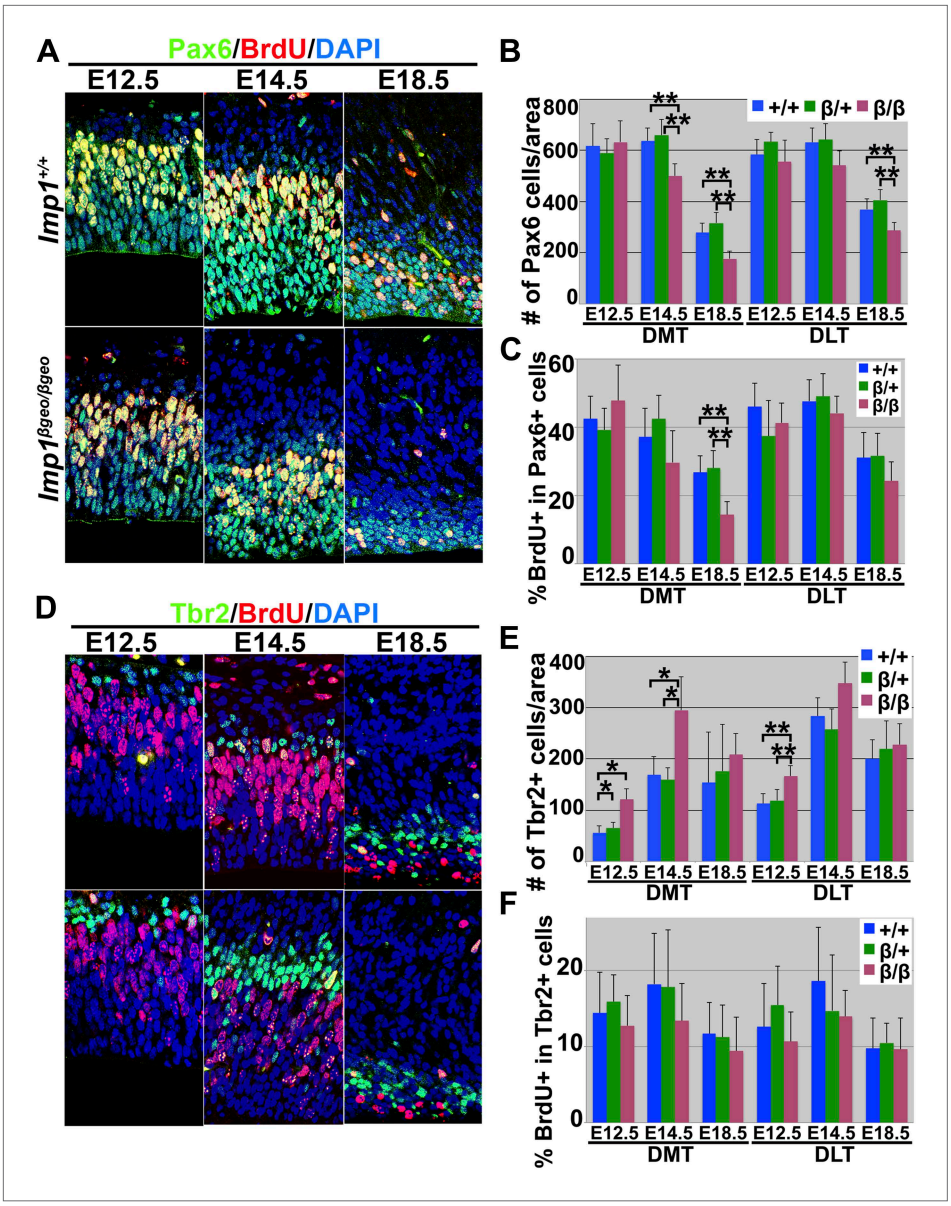

Figure 3. *Imp1* deficiency leads to precocious maturation of Pax6+ stem cells into Tbr2+ intermediate neuronal progenitors in the dorsal telencephalon. (**A**–**C**) *Imp1* deficiency significantly reduced the number of Pax6+ neural stem cells in the dorsomedial telencephalon (DMT) at E14.5 and E18.5, and in the dorsolateral telencephalon (DLT) at E18.5 (**p<0.05; mean ± SD for 3–4 mice/genotype at each stage with 6–8 sections/brain). *Imp1* deficiency significantly reduced the percentage of Pax6+ neural stem cells that were BrdU+ in E18.5 DMT (**p<0.05; mean ± SD for 3–4 mice/genotype at each stage with 6–7 sections/brain). (**D**–**F**) *Imp1* deficiency transiently increased the number of Tbr2+ intermediate progenitors in the DMT at E12.5 and E14.5, and in the DLT at E12.5 (*p<0.01; mean ± SD for 3–5 brains/genotype at each stage with 6–8 sections/brain). *Imp1* deficiency did not significantly affect the percentage of Tbr2+ cells that were also BrdU+ (mean ± SD for 3–4 mice/genotype with six sections/brain).
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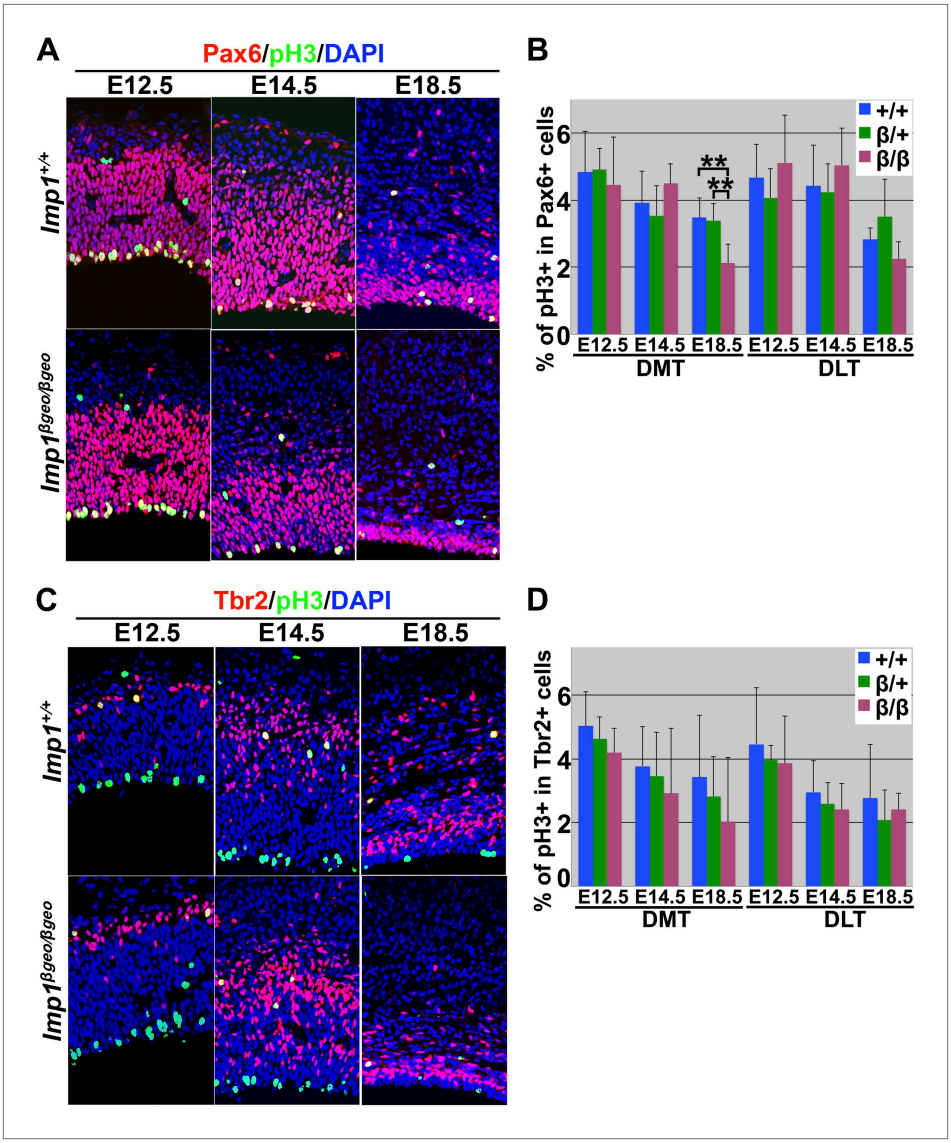

Figure 3—figure supplement 1. *Imp1* deficiency significantly reduced the percentage of proliferating Pax6+ neural stem cells in E18.5 dorsomedial telencephalon (DMT). (**A**–**B**) DMT sections stained with antibodies against Pax6 and phospho-Histone H3 (pH3), a marker of mitotic cells. (**B**) *Imp1* deficiency significantly reduced the percentage of Pax6+ neural stem cells that were pH3+ in E18.5 DMT but not at E12.5 or E14.5, or in DLT (**p<0.05; mean ± SD for 3–4 brains/genotype at each stage with 4–7 sections/brain). (**C**–**D**) DMT sections stained with antibodies against Tbr2 and phospho-Histone H3. (**D**) *Imp1* deficiency did not significantly affect the percentage of Tbr2+ cells that were also pH3+ (mean ± SD for 3–4 brains/genotype with six sections/brain).

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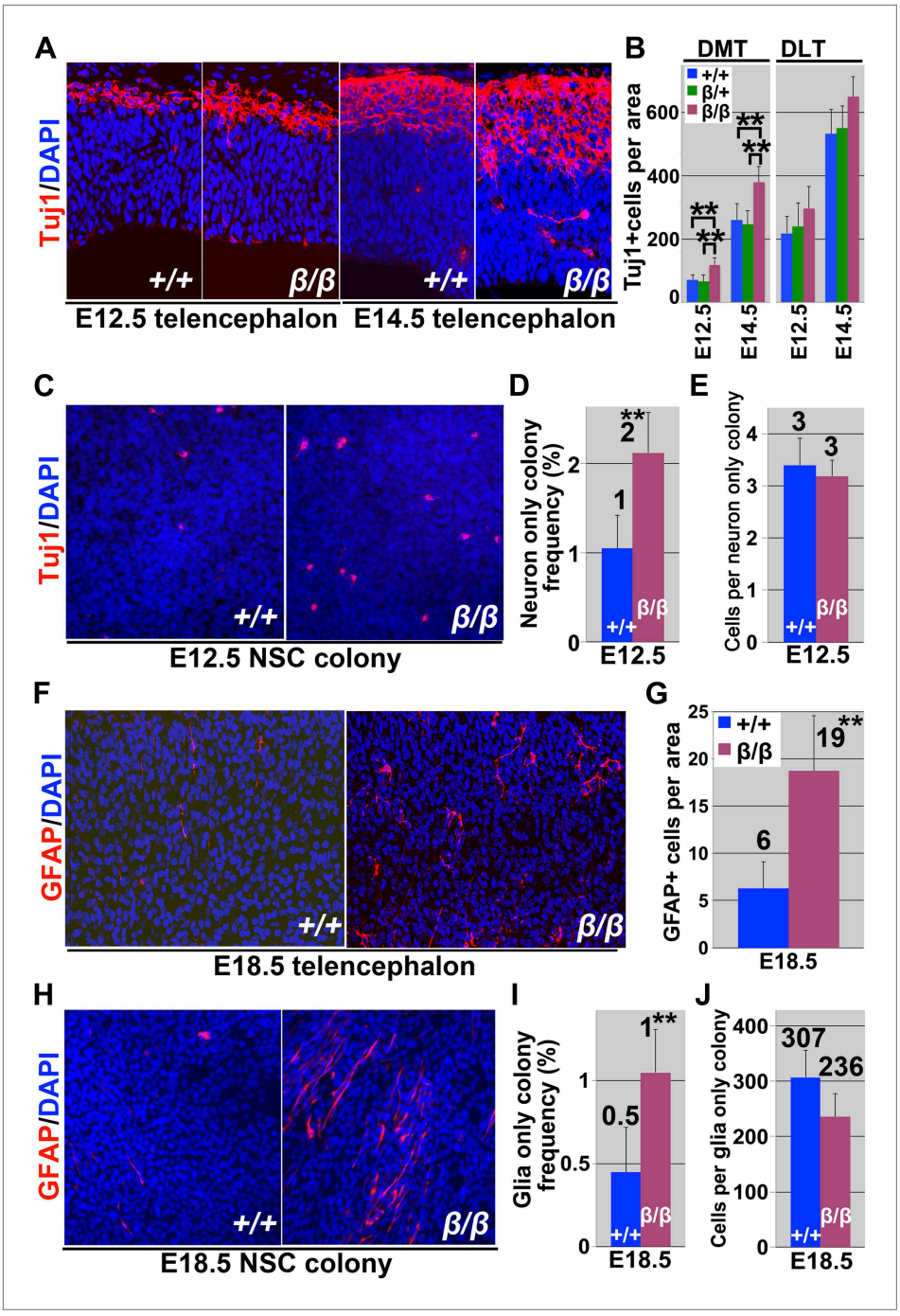

Figure 4. IMP1 prevents premature neuronal and glial differentiation by stem cells in the dorsal telencephalon. (**A**) Dorsomedial telencephalon sections from E12.5 or E14.5 control (+/+) or *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) embryos were stained with an antibody against the neuronal marker Tuj1. (**B**) The number of Tuj1+ neurons per section was significantly increased in *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) DMT as compared to littermate controls (+/+: *Imp1*$^{+/+}$ or β/+: *Imp1*$^{β\text{-}geo/+}$) at E12.5 and E14.5 (**p<0.05; mean ± SD for four brains/genotype with 4–6 sections/brain). (**C**–**E**) E12.5 dorsal telencephalon cells were cultured adherently for 9 days at clonal density. (**C**) Tuj1+ neurons were significantly more common in multipotent colonies from *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) as compared to *Imp1*$^{+/+}$ (+/+) mice. (**D**) Significantly more neuron-only colonies were formed by *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) as compared to *Imp1*$^{+/+}$ (+/+) telencephalon cells; however, the number of cells within control (+/+) or *Imp1*$^{β\text{-}geo/β\text{-}geo}$ (β/β) neuron-only colonies did not significantly differ (**E**; **p<0.05; mean ± SD for three independent experiments). (**F**) Dorsal telencephalon sections from E18.5 control (+/+) or

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Imp1$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) embryos were stained with an antibody against GFAP. (**G**) The number of GFAP+ astrocytes per section was significantly increased in the *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) dorsal telencephalon as compared to littermate controls (+/+) at E18.5 (**p<0.05; mean ± SD for four brains/genotype with 6–8 sections/brain). (**H**–**J**) E18.5 dorsal telencephalon cells were cultured adherently for 9 days at clonal density. (**H**) GFAP+ astrocytes were more common in multipotent colonies from *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) as compared to *Imp1*$^{+/+}$ (+/+) mice. (**I**) Significantly more glia-only colonies were formed by *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) as compared to *Imp1*$^{+/+}$ (+/+) telencephalon cells; however, the number of cells within control (+/+) or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) glia-only colonies did not significantly differ (**J**; **p<0.05; mean ± SD for three independent experiments).

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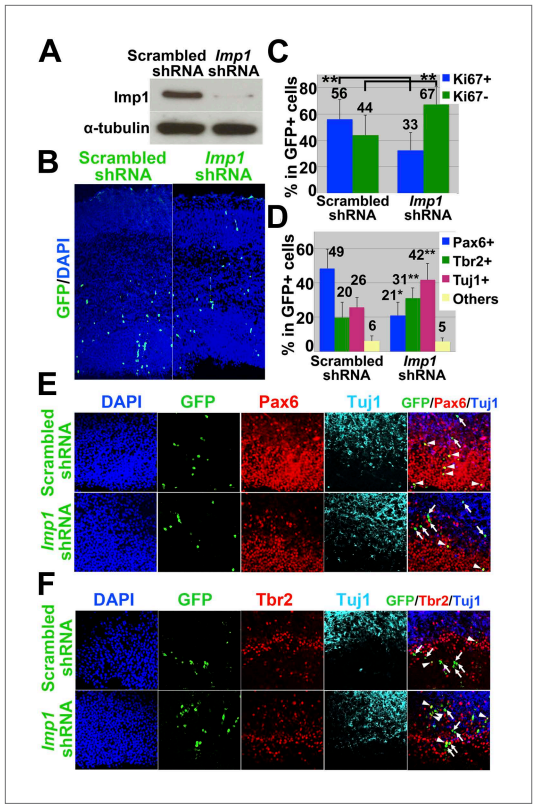

Figure 5. In utero knockdown of *Imp1* cell-autonomously reduces cell proliferation and accelerates differentiation of neural stem/progenitor cells. (**A**) Western blot of neurospheres cultured from E14.5 wild-type telencephalon cells infected with lentivirus bearing either control (scrambled) shRNA or *Imp1* shRNA. *Imp1* shRNA reduced IMP1 expression. (**B** and **C**) Viruses expressing either control or *Imp1* shRNA were injected into the telencephalic ventricles of E14.5 wild-type mice, infecting a small percentage of cells that could be identified based on GFP expression. Brains were fixed at E17.5 and dorsomedial telencephalon sections were immunostained with antibodies against GFP and Ki67. (**B**) Low magnification view of sections through the dorsal telencephalon including VZ (apical; bottom) and differentiated cell layers (basal; top). In *Imp1* shRNA infected telencephalon, GFP+ cells were more likely to be found basally as compared to control shRNA infected cells, suggesting that *Imp1* shRNA promoted the differentiation of infected cells. (**C**) The percentages of GFP+ (infected) cells that were Ki67+ (dividing) or Ki67- at E17.5. *Imp1* shRNA infection significantly reduced the percentage of GFP+ cells that were Ki67+ (**p<0.05; mean ± SD for four experiments). (**D**–**F**) Viruses expressing either control or *Imp1* shRNA were injected into the telencephalic ventricles of E12.5 wild-type mice. Brains were fixed at E15.5 and dorsomedial telencephalon sections were immunostained to assess the differentiation of GFP+ cells. (**D**) *Imp1* shRNA significantly increased the percentage of GFP+ cells that were Tbr2+ or Tuj1+ and significantly

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reduced the percentage that were Pax6+ (**p<0.05; mean ± SD for five experiments). (**E**) Triple immunostaining with antibodies against GFP, Pax6, and Tuj1. GFP+/Pax6+ cells (yellow cells in merged image) are marked with arrowheads, and GFP+/Pax6- cells are indicated with arrows. (**F**) Triple immunostaining with antibodies against GFP, Tbr2, and Tuj1. GFP+/Tbr2+ cells (yellow cells in merged image) are marked with arrowheads and GFP+/Tbr2- cells are indicated with arrows.

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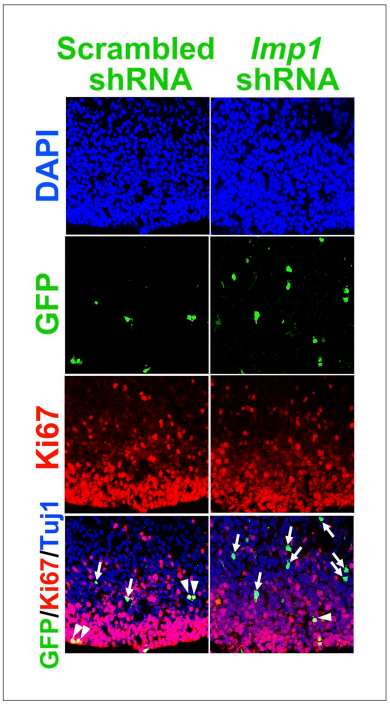

Figure 5—figure supplement 1. *Imp1* knockdown in neural stem cells reduces cellular proliferation. Viruses expressing either control (scrambled) shRNA or *Imp1* shRNA were injected into the telencephalic ventricles of E14.5 wild-type mice. Brains were fixed at E17.5 and dorsomedial telencephalon sections were immunostained with antibodies against GFP and Ki67. GFP+/Ki67+ dividing, virally infected cells (yellow cells in merged image) are indicated with arrowheads, and GFP+/Ki67- non-dividing virally infected cells (green cells in merged image) are indicated with arrows.

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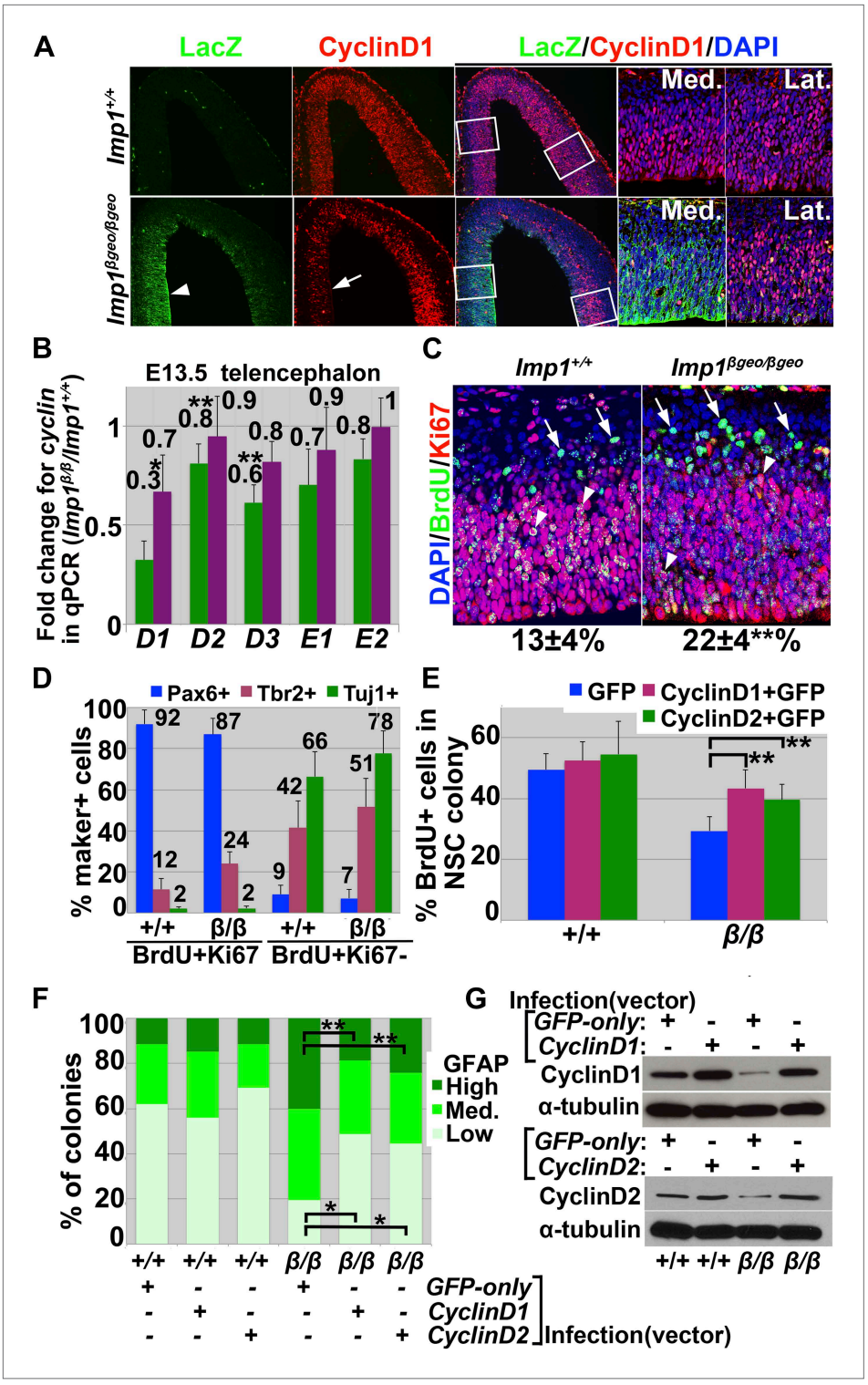

Figure 6. *Imp1* deficiency reduced cyclin D expression and accelerated cell cycle exit in the dorsal telencephalon. (**A**) Sections of E13.5 wild-type or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ dorsal telencephalon were immunostained with antibodies against Cyclin D1 and LacZ. Cyclin D1 expression was reduced relative to control in *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ DMT (see arrow) where strong LacZ immunostaining indicated the highest levels of *Imp1* expression (see arrowhead). In contrast, Cyclin D1 immunostaining was retained in *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ DLT, where lacZ immunostaining was weak. Higher magnification images on the right show boxed areas from low magnification images. (**B**) qPCR analysis of *cyclin D* and *cyclin E*

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transcripts in dorsomedial (green bar) and dorsolateral (purple bar) telencephalon from E13.5 mice. Each bar represents the fold change in *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$/wild-type (error bars represent SD, four brains/genotype; *p<0.01, **p<0.05). (**C**) Dorsal telencephalon sections from E14.5 *Imp1*$^{+/+}$ or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ mice that had been administered a single pulse of BrdU at E13.5 were stained with anti-BrdU and anti-Ki67 antibodies. Cells that exited the cell cycle after BrdU incorporation were BrdU+Ki67- (green; arrows) while cells that continued to divide were BrdU+Ki67+ cells (yellow; arrowheads). The *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ telencephalon had a significantly higher percentage of BrdU+Ki67- cells (22 ± 4%; **p<0.05; mean ± SD for four brains/genotype; 4–6 sections/brain). (**D**) Most BrdU+Ki67+ cells expressed Pax6 and most BrdU+Ki67- cells were either Tbr2+ or Tuj1+. Single channel images are presented in ***Figure 6—figure supplement 1***. (**E**–**G**) E18.5 wild-type (+/+) or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) neural stem cells were infected with either *GFP-only* control retrovirus, *cyclin D1-GFP* retrovirus, or *cyclin D2-GFP* retrovirus and cultured. Within the resulting neural stem cell colonies, cell proliferation was assessed by BrdU incorporation (**E**), glial differentiation was assessed based on levels of GFAP staining (**F**), and Cyclin D1 or Cyclin D2 expression was examined by western blot (**G**). *Imp1* deficiency reduced Cyclin D1 or Cyclin D2 expression and neural stem cell proliferation and increased gliogenesis. These proliferation and premature gliogenesis phenotypes were partially rescued by cyclin D1 or cyclin D2 over-expression (three experiments; *p<0.01, **p<0.05).

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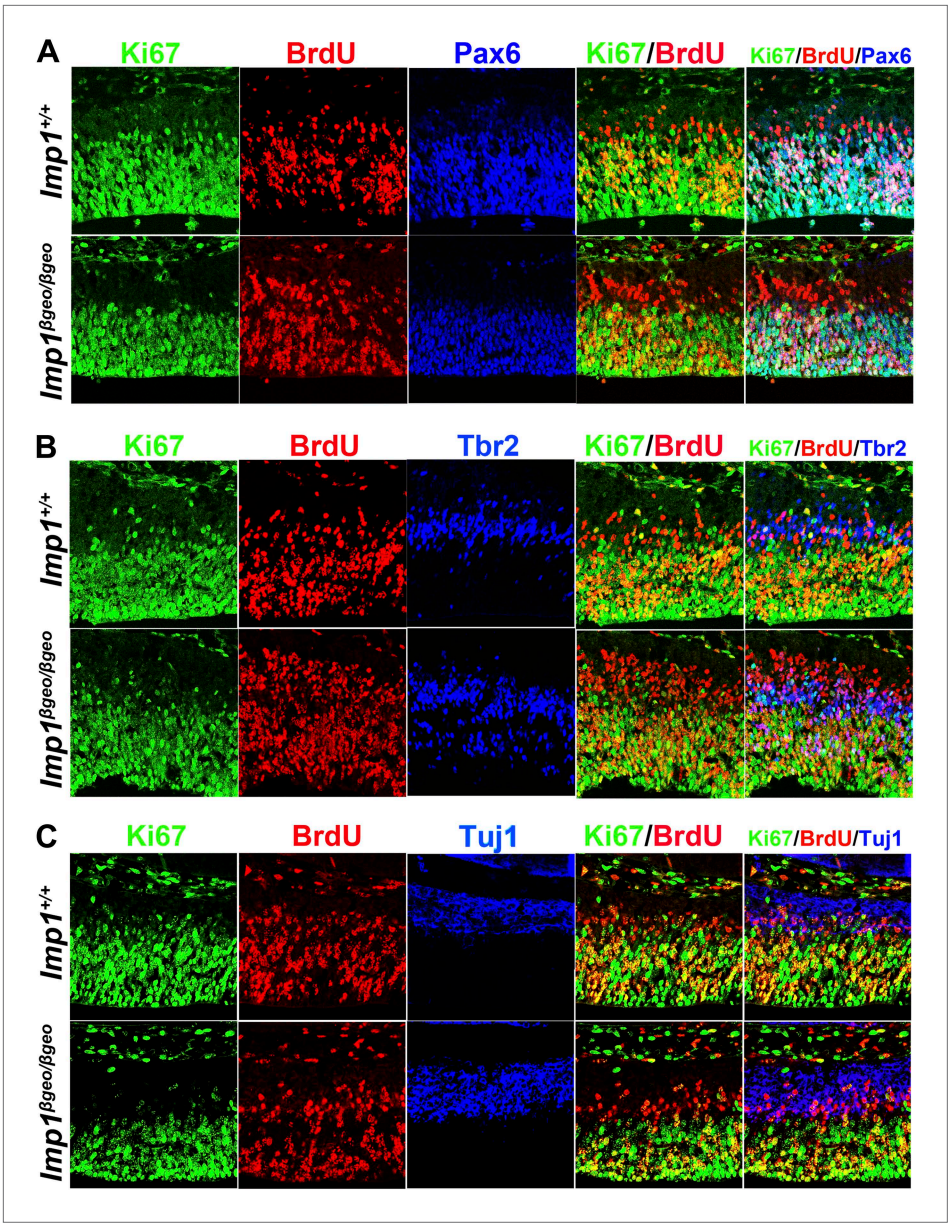

Figure 6—figure supplement 1. BrdU+/Ki67+ cells were Pax6+ while BrdU+/Ki67- cells were Tbr2+ or Tuj1+ in E14.5 dorsomedial telencephalon. Brains were dissected from E14.5 wild-type or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ mice after a 24 hr pulse of BrdU. Coronal sections were immunostained with antibodies against BrdU, Ki67, and Pax6 (**A**), Tbr2 (**B**), or Tuj1(**C**). Most BrdU+Ki67+ cells (yellow cells in Ki67/BrdU images) expressed Pax6 and most BrdU+Ki67- cells (red cells in Ki67/BrdU images) were either Tbr2+ or Tuj1+.

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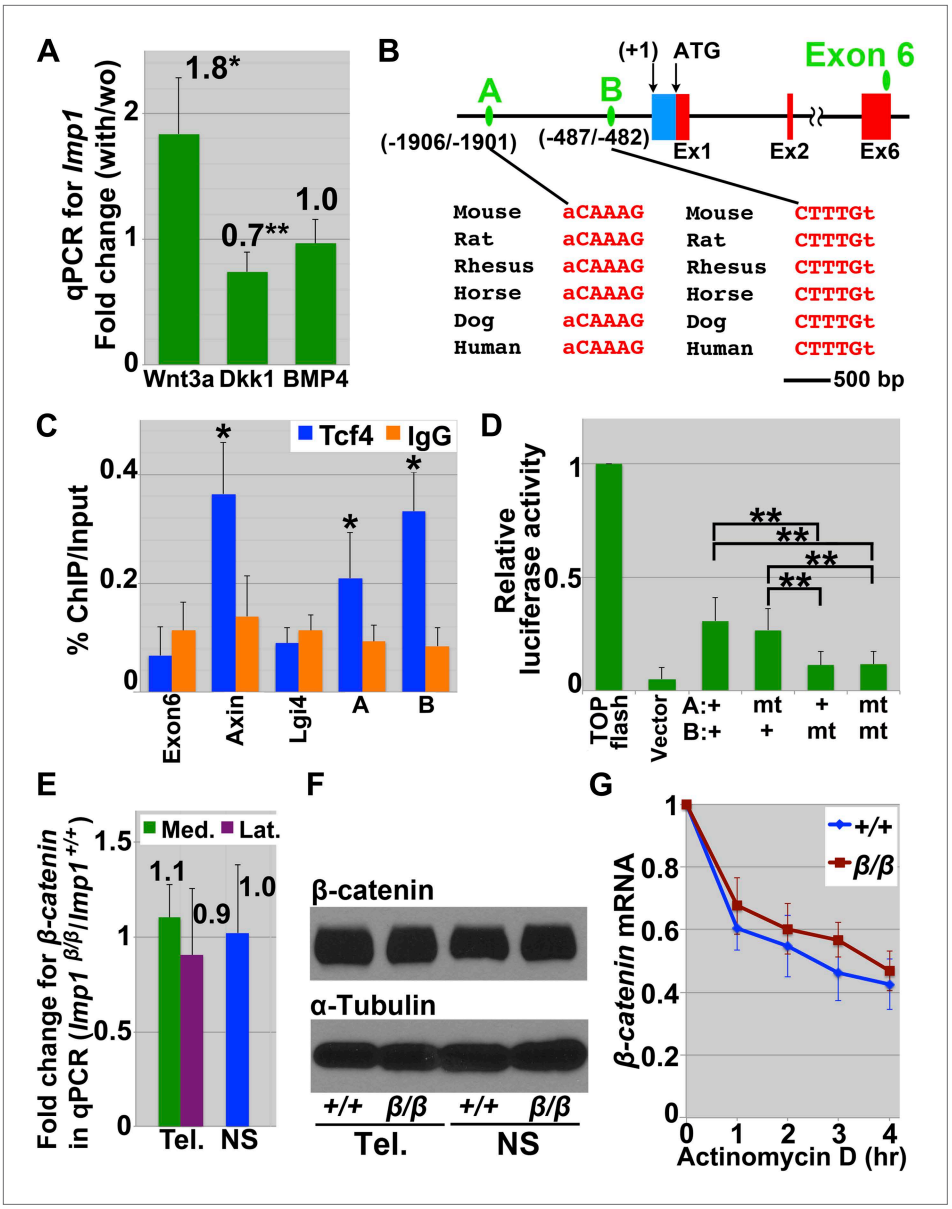

Figure 6—figure supplement 2. Canonical Wnt signaling promotes *Imp1* expression. (**A**) In E12.5 dorsolateral telencephalon explants cultured for 12 hr, *Imp1* transcript levels were significantly increased by Wnt-3a, decreased by Dkk-1, and unaffected by BMP4. Each bar represents the ratio of *Imp1* transcript levels in the presence/absence of the recombinant proteins (error bar represent SD, 3–5 independent experiments; *p<0.01; **p<0.05). (**B**) Within 3 kb upstream of the transcription starting site (TSS, designated as +1), two sequences are conserved across species and match consensus binding sites for TCF/LEF transcription factors (A and B; green ovals). The 5′-untranslated region of *Imp1* is shown as a blue box and exons are shown as red boxes. We used exon 6 sequence as an internal negative control during chromatin immunoprecipitation. (**C**) Chromatin immunoprecipitation of TCF4 from E12.5 wild-type CNS neurospheres. Two sites in the promoters of *Imp1* (sites A and B in panel **B**), and *Axin-2* (a positive control) were significantly enriched by TCF4 immunoprecipitation (blue bars) compared to IgG immunoprecipitation (orange bars). No enrichment was detected for exon 6 of *Imp1* or *Lgi4* (negative controls). (**D**) Luciferase assay performed in P19 embryonal carcinoma cells transfected with plasmids either containing intact *Imp1* enhancer/promotor (A+/B+), site A eliminated (A mt /B+), site B eliminated (A+/B mt), or both site A/site B eliminated (A mt/B mt). TOP-flash or empty vector are included as positive or negative controls. Site B elimination significantly reduced luciferase activity whereas site A elimination had little effect (error bar represent SD of three independent experiments; **p<0.05). (**E**) *Imp1* deficiency did not affect the levels of *β-catenin* (*Ctnnb1*) transcripts in E13.5 dorsomedial (green bar) or dorsolateral telencephalon (purple bar), or in E13.5 neurospheres (blue bar).

Figure 6—figure supplement 2. Continued on next page

Figure 6—figure supplement 2. Continued

Error bars represent SD; three brains/genotype, three independent experiments. (**F**) *Imp1* deficiency did not affect ß-catenin protein levels in E13.5 dorsal telencephalon or neurospheres. (**G**) *Imp1* deficiency did not significantly affect the stability of *ß-catenin* mRNA after treatment with Actinomycin D in neurospheres cultured from E13.5 control (blue) or $Imp1^{\beta\text{-}geo/\beta\text{-}geo}$ (red) dorsal telencephalons (error bars represent SD; three independent experiments).

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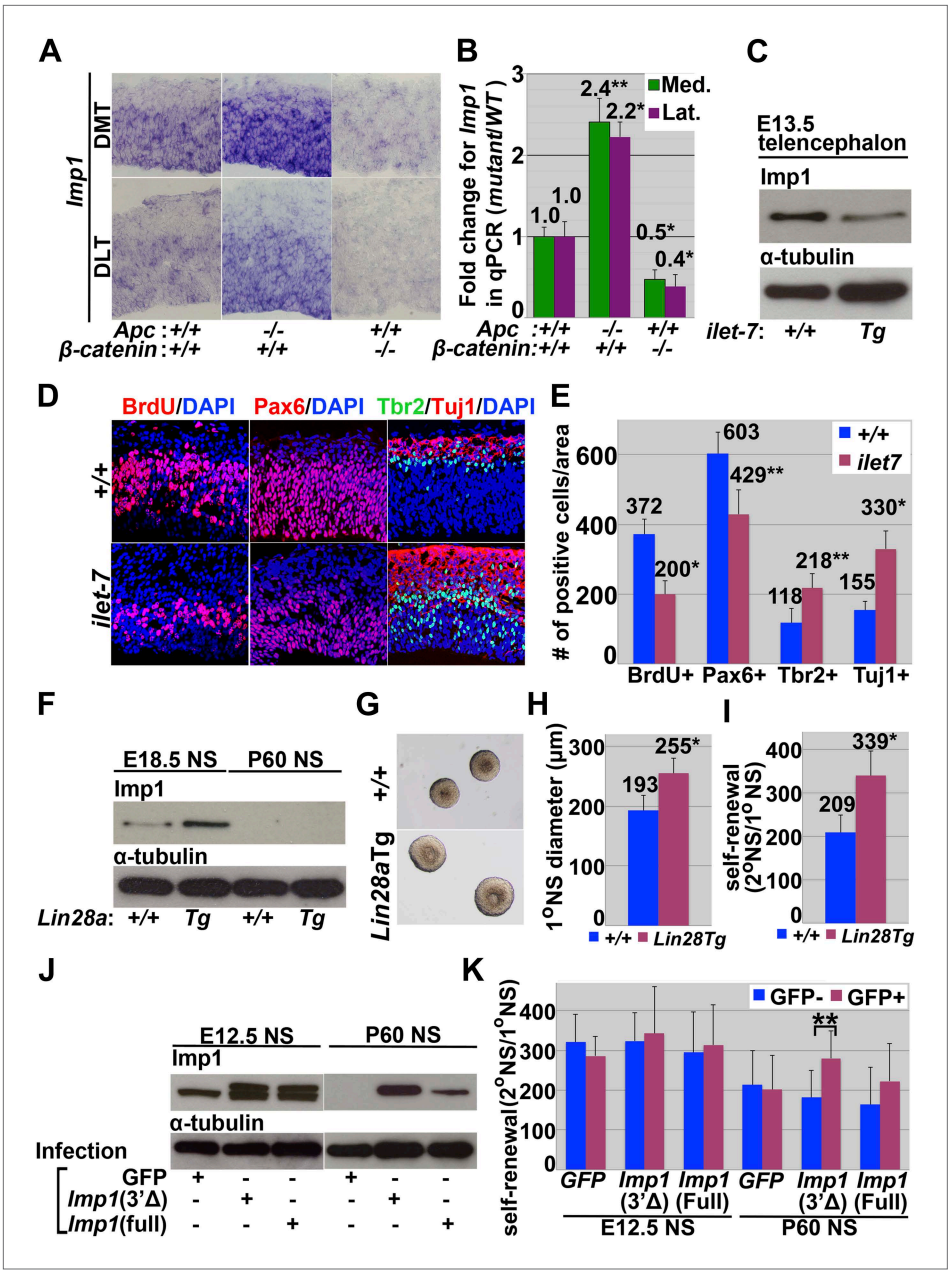

Figure 7. Canonical Wnt signaling promotes, and *let-7* inhibits, *Imp1* expression. (**A**–**B**) *Imp1* transcript levels were elevated in E14.5 dorsal telencephalon of *Apc*-deficient mice, and reduced in *ß-catenin* (*Ctnnb1*)-deficient mice by both in situ hybridization (**A**) and qPCR (**B**). Bars represent fold change in *Imp1* transcript levels in dorsomedial (green bars) and dorsolateral (purple bars) telencephalon of the indicated mutant mice/wild-type controls (*p<0.01, **p<0.05; error bar represents SD, 3–4 brains/genotype). (**C**) Western blot for IMP1 or α-tubulin in E13.5 telencephalon cells isolated from doxycycline administered wild-type (+/+) or *let-7* inducible transgenic mice (*ilet-7* Tg). (**D**–**E**) Dorsomedial telencephalon sections were prepared from doxycycline administered E13.5 wild-type (+/+) or *let-7* inducible transgenic mice (*ilet-7* Tg). Induction of *let-7* transgene expression significantly reduced the number of proliferating cells (assessed by a 1 hr pulse of BrdU) and the frequency of Pax6+ cells, and increased the numbers of Tbr2+ intermediate progenitors and Tuj1+ neurons (*p<0.01, **p<0.05; error bar represents SD, 3–4 mice/genotype). (**F**–**I**) E18.5 dorsal telencephalon cells or P60 SVZ cells were isolated from doxycycline administered wild-type (+/+) or *Lin28a* inducible transgenic mice (*Lin28a* Tg) and cultured as neurospheres. (**F**) Western blot for IMP1 or α-tubulin. IMP1 protein expression was elevated in *Lin28a* transgenic cells relative to control at E18.5 and not detected in either cells at P60. (**G**–**I**) *Lin28a* induction significantly increased the size of E18.5 neurospheres (**G** and **H**) and their self-renewal potential (**I**; *p<0.01; mean ± SD for four experiments). (**J**–**K**) E12.5 wild-type dorsal

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telencephalon or P60 wild-type SVZ cells were infected with *GFP-only* control retrovirus (GFP), 3′UTR truncated 3XFLAG-*Imp1-GFP* retrovirus (3**Δ**), or full length 3XFLAG *Imp1-GFP* retrovirus (full). Truncated *Imp1* lacked *let-7* binding sites in the 3′ UTR. (**J**) Over-expression of truncated 3XFLAG-*Imp1-GFP* increased IMP1 protein expression more efficiently than full length 3XFLAG *Imp1-GFP* over-expression at P60 when *let-7* expression is high. The smaller band corresponds to endogenous IMP1 while the larger band corresponds to FLAG-tagged IMP1. (**K**) Only 3′ UTR truncated 3XFLAG *Imp1-GFP* over-expression significantly increased the self-renewal of neurospheres relative to uninfected cells at P60.

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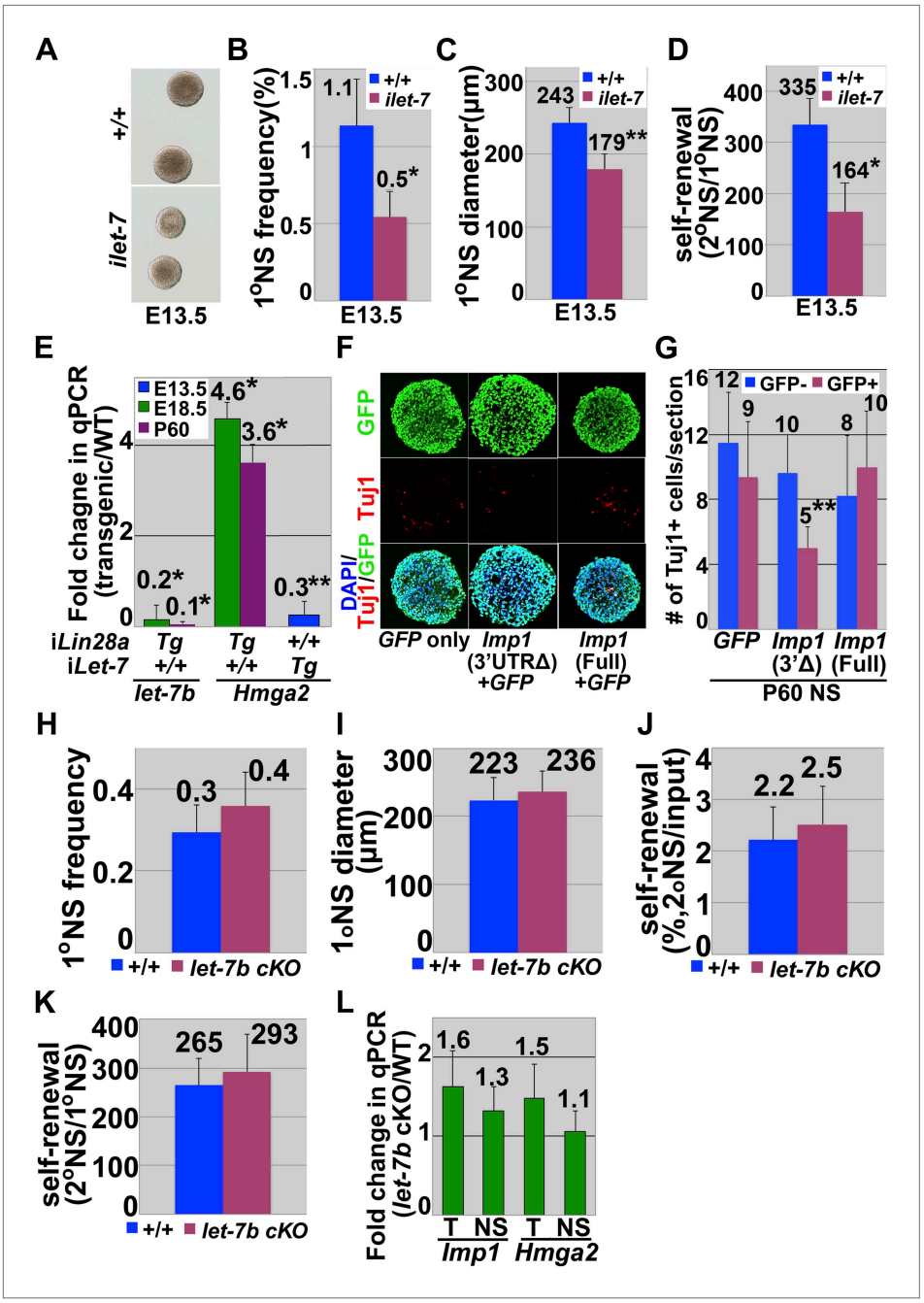

Figure 7—figure supplement 1. *let-7* over-expression inhibits neural stem cell self-renewal while *Imp1* over-expression inhibits neurogenesis. Conditional deletion of *let-7b/c2* did not affect neural stem cell function or *Imp1* or *Hmga2* expression. (**A**–**D**) Neurospheres were cultured from E13.5 dorsal telencephalons isolated from doxycycline administered wild-type (+/+) or *let-7* inducible transgenic mice (*ilet-7*). Induction of *let-7* significantly reduced the percentage of cells that formed multilineage neurospheres (**B**), neurosphere size (**C**), and self-renewal potential (the number of cells from individual primary neurospheres that formed multilineage secondary neurospheres upon subcloning) (**D**; mean ± SD for three experiments). (**E**) In E18.5 or P60 *Lin28a* Tg neurospheres, *let-7b* expression was significantly reduced and *Hmga2* transcript expression was significantly increased compared to control. *Hmga2* transcript expression was significantly reduced in E13.5 *ilet-7* dorsal telecephalon compared to control. (**F**–**G**) P60 wild-type SVZ cells were infected with *GFP-only* control retrovirus (GFP), 3′-UTR truncated 3XFLAG-*Imp1-GFP* retrovirus (3Δ), or 3′-UTR containing 3XFLAG *Imp1-GFP* retrovirus (full-length). Only 3′-UTR truncated *Imp1* over-expression significantly reduced the number of Tuj1+ neurons per section (mean ± SD for four experiments).

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(**H**–**L**) Dorsal telencephalon cells from E18.5 wild-type (+/+) or *Nestin-Cre; let-7b/c2* conditional mutant (*let-7b/c2* cKO) mice were cultured non-adherently. *let-7b/c2* deficiency did not affect the percentage of cells that formed multipotent neurospheres (**H**), neurosphere size (**I**), or neural stem cell self-renewal (the number or percentage of cells from individual primary neurospheres that formed multilineage secondary neurospheres upon subcloning; **J** and **K**). Expression levels of *Imp1* or *Hmga2* transcripts assessed by qPCR were also not significantly altered by *let-7b/c2* deficiency (**L**) (mean ± SD for three experiments).

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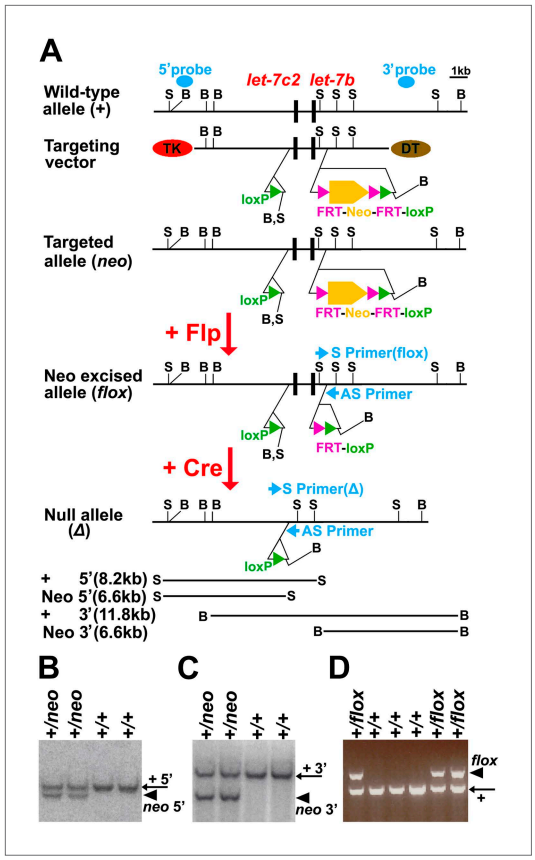

Figure 7—figure supplement 2. Generation of a conditional mutant allele of *let-7b* and *let-7c2* (*let-7b/c2*^{fl}) by gene targeting. (**A**) In the wild-type genome sequences that encode primary *let-7b* microRNA (pri-*let-7b*) and primary *let-7c2* microRNA (pri-*let-7c2*) are closely linked (black boxes). A targeting vector was designed to flank these sequences with loxP elements (green triangles), flippase recognition sites (FRT, purple triangles), and a neomycin resistance cassette (Neo, orange hexagon). For negative selection of ES cells that randomly inserted the targeting construct, *thymidine kinase* (TK) and *diphtheria toxin* (DT) were included at both ends of the targeting vector. Correct targeting in ES cells was confirmed by Southern blot using 5′ and 3′ probes (blue ovals). After mice were generated from correctly targeted ES clones, the neomycin resistance cassette was eliminated by mating the mice with *Actin-Flpe* mice (***Rodriguez et al., 2000***), and confirmed by PCR with primer pairs (blue arrows). S: ScaI endonuclease sites, B: BamHI endonuclease sites. (**B** and **C**) Correctly targeted ES cell clones were confirmed by Southern blot. (**B**) With 5′-probe, the wild-type allele (+, arrow) gave a band of 8.2 kb and the mutant allele (*neo*, arrowhead) gave a band of 6.6 kb. (**C**) With 3′-probe, the wild-type allele (+, arrow) gave a band of 11.8 kb and the mutant allele (*neo*, arrowhead) gave a band of 6.6 kb. (**D**) PCR genotyping of the progeny from *let-7b/c2*^{neo} and *Actin-Flpe* mice. The

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wild-type allele (+, arrow) yielded a 317 bp band and the *floxed* allele (*flox*, arrowhead) yielded a 408 bp band.

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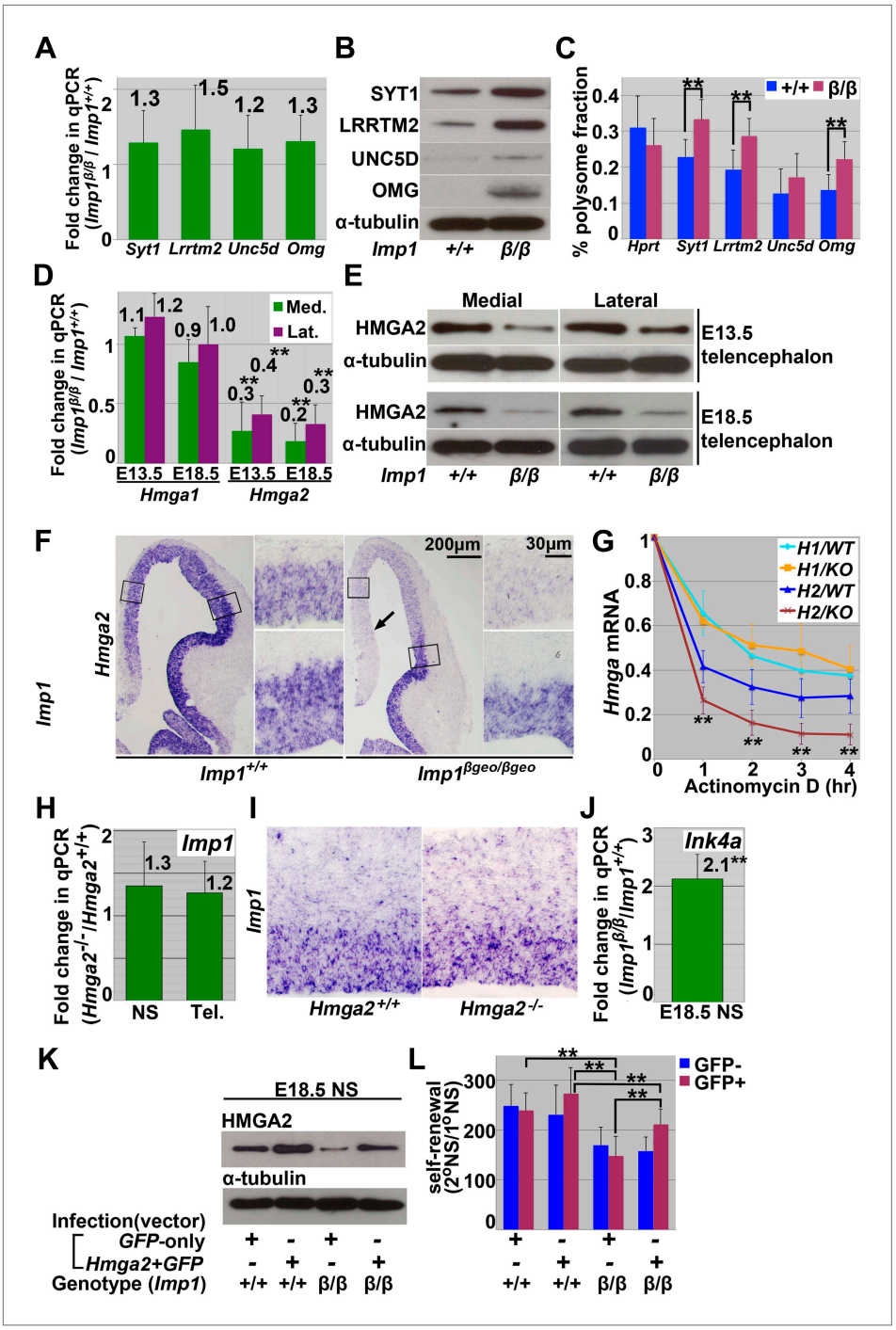

Figure 8. *Imp1* acts post-transcriptionally and cell autonomously to negatively regulate the expression of gene products associated with differentiation and to promote the expression of self-renewal genes, including *Hmga2*. (**A**–**B**) *Imp1* deficiency did not affect the levels of *synaptotagmin1* (*Syt1*), *Leucine rich repeat transmembrane neuronal 2* (*Lrrtm2*), *Unc5d*, or *Oligodendrocyte myelin glycoprotein* (*Omg*) transcripts by qPCR in E13.5 dorsal telencephalon-derived neurospheres; however, *Imp1* deficiency did increase the levels of SYT1, LRRTM2, UNC5d, and OMG proteins. (**C**) Neurosphere lysates from E13.5 wild-type (+/+) or $Imp1^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) dorsal telencephalon were fractionated in a 10–50% sucrose gradient (***Figure 8—figure supplement 8***) and the transcripts in the polysome fraction were assessed by qPCR. *Synaptotagmin1* (*Syt1*), *Leucine rich repeat transmembrane neuronal 2* (*Lrrtm2*), and *Oligodendrocyte myelin glycoprotein* (*Omg*) transcripts were significantly enriched in the polysome fractions in *Imp1* deficient cells relative to control cells (**p<0.05; error bars represent SD from three independent

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experiments). (**D**) *Hmga2*, but not *Hmga1*, transcript levels were significantly reduced in *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ dorsomedial (green bar) and dorsolateral telencephalon (purple bar) (*p<0.01, **p<0.05; error bars represent SD, 3–4 brains/genotype). (**E**) *Imp1* deficiency reduced HMGA2 levels in dorsomedial, and dorsolateral telencephalon. (**F**) *Imp1* deficiency reduced *Hmga2* transcript levels (by in situ hybridization; purple) in dorsomedial and dorsolateral telencephalon from E13.5 mice. Higher magnification views of the boxed regions of dorsomedial (upper) or dorsolateral (lower) telencephalons are shown to the right of lower magnification images. Note that *Hmga2* expression declined to a greater extent in the dorsomedial telencephalon (arrow) where *Imp1* expression is highest and did not decline in the ventral telencephalon where *Imp1* is not expressed at this stage (see ***Figure 1D***). *Imp1* is thus required cell autonomously in the dorsal telencephalon to maintain *Hmga2* expression. (**G**) *Hmga2*, but not *Hmga1*, transcript levels were significantly (**p<0.05) reduced after Actinomycin D treatment in *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (red) relative to wild-type (blue) neurospheres cultured from E13.5 dorsal telencephalon. Error bars represent SD in three experiments. (**H**–**I**) *Hmga2* deficiency did not affect *Imp1* transcript levels in E13.5 telencephalon cells by qPCR (**H**) or in situ hybridization (**I**) (three independent experiments). (**J**) *Ink4a* transcript levels were significantly elevated by *Imp1* deficiency in neurospheres cultured from E18.5 wild-type or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ dorsal telencephalon (**p<0.05; error bars represent SD, 3–4 mice/genotype). (**K** and **L**) E18.5 wild-type (+/+) or *Imp1*$^{\beta\text{-}geo/\beta\text{-}geo}$ (β/β) dorsal telencephalon cells were infected with *GFP-only* control retrovirus (GFP) or *Hmga2-GFP* retrovirus. *Imp1* deficiency reduced HMGA2 expression and neural stem cell self-renewal but both were restored by *Hmga2* over-expression.

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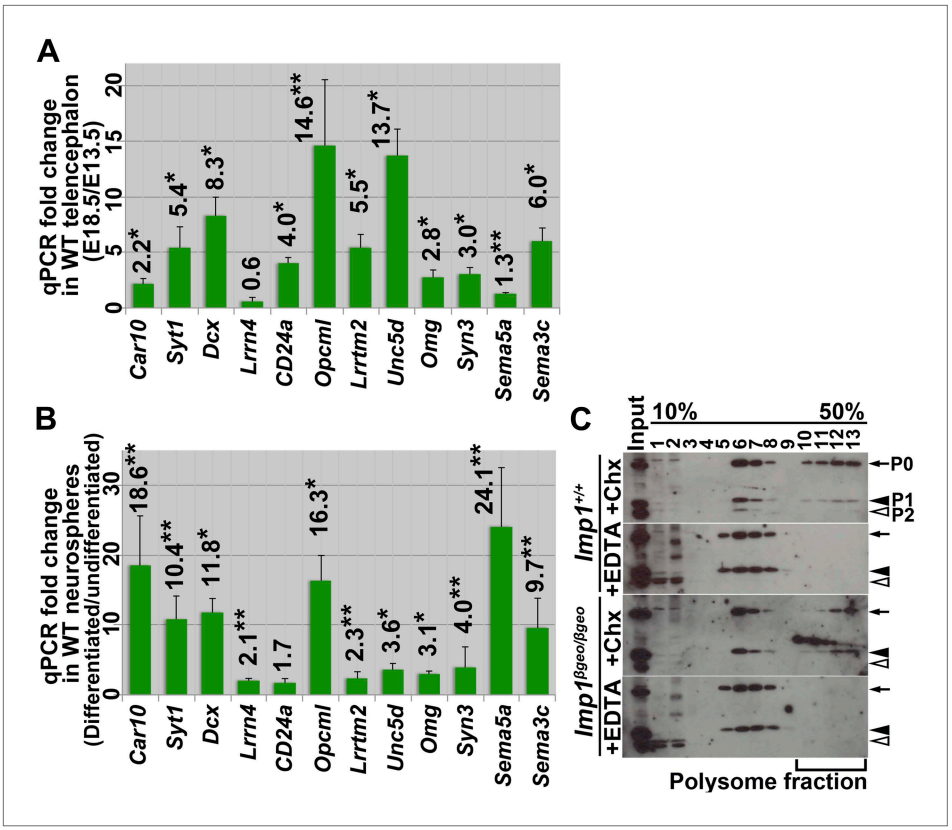

Figure 8—figure supplement 1. Multiple mRNAs bound by IMP1 increased their expression during brain development and neural differentiation. (**A**) A number of mRNAs bound by IMP1 encode gene products associated with differentiated neurons and glia. The levels of these mRNAs were compared by qPCR in dorsal telencephalons from E13.5 and E18.5 wild-type mice. Bars represent the ratio of transcript levels in E18.5/E13.5 telencephalon (*p<0.01, **p<0.05; three mice/time point; error bars always represent SD). The expression levels of these mRNAs generally increased during development as would be expected for gene products expressed by differentiated cells. (**B**) The levels of these mRNAs were also compared by qPCR in neurospheres before and after differentiation. Neurospheres were cultured from E13.5 wild-type dorsal telencephalons. Bars represent the ratio of transcript levels in differentiated/undifferentiated neurospheres (*p<0.01, **p<0.05; three independent experiments). (**C**) Cell lysates from E13.5 wild-type or *Imp1*$^{β\text{-}geo/β\text{-}geo}$ neurospheres were fractionated in a 10–50% sucrose gradient and the distribution of ribosomal proteins was assessed by immunoblotting against ribosomal P0, P1, and P2 antigens. RNAs were isolated from the input or polysomal fraction in the cycloheximide treated gradient (+Chx, fractions #10–13) and subjected to qPCR in ***Figure 7C*** to quantitate the relative abundance of mRNAs in the polysomal fraction. In parallel, EDTA was included in the gradient to dissociate ribosomal complexes to assess the position of the polysomal fraction.

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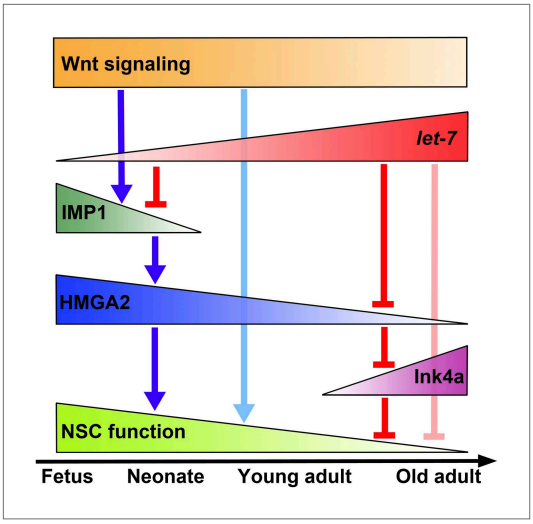

Figure 9. Schematic showing a network of heterochronic genes that regulate temporal changes in CNS stem cell properties from fetal development throughout adulthood. A network of heterochronic genes changes with age, leading to temporal changes in stem cell properties from fetal development throughout adulthood. The promotion of expression/function is indicated as blue arrows and negative regulation is indicated as red bars. In fetal neural stem cells, *Imp1* expression is promoted transcriptionally by canonical Wnt signaling and inhibited post-transcriptionally by *let-7*. IMP1 expression is reduced by increasing *let-7* expression in late fetal development and transcriptionally silenced postnatally. HMGA2 expression is high in early development but declines with time in response to declining IMP1 and increasing *let-7*. Increasing *let-7* expression contributes to the reduction in HMGA2 expression over time during adulthood. HMGA2 negatively regulates the expression of Ink4a/Arf. Declining expression of HMGA2 during adulthood allows the expression of these gene products to increase during aging. Overall, neural stem cell function declines over time. A decline in Wnt signaling during aging also contributes to these effects (***Seib et al., 2013***).

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